Rapid Neutrophil Response Controls Fast-Replicating Intracellular Bacteria but Not Slow-Replicating *Mycobacterium tuberculosis*

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Being one of the first cells to invade the site of infection, neutrophils play an important role in the control of various bacterial and viral infections. In the present work, the contribution of neutrophils to the control of infection with different intracellular bacteria was investigated. Mice were treated with the neutrophil-depleting monoclonal antibody RB6-8C5, and the time course of infection in treated and untreated mice was compared by using intracellular bacterial species and strains varying in virulence and replication rate. The results indicate that neutrophils are crucial for the control of fast-replicating intracellular bacteria, whereas early neutrophil effector mechanisms are dispensable for the control of the slow-replicating *Mycobacterium tuberculosis*.

Neutrophilic polymorphonuclear granulocytes (neutrophils) are among the first cells attracted to the site of infection [1]. In support of local tissue macrophages, neutrophils play a critical role in the restriction of microbial replication and spread early after pathogen entry. To do so, neutrophils exhibit a dual function. First, neutrophils phagocytose pathogens efficiently [1] and exhibit potent microbicidal activity mediated by granular enzymes, antimicrobial peptides and proteins, and reactive intermediates of oxygen and nitrogen [2–6]. As one of the most efficient phagocytic cells of the immune system, neutrophils restrict the initial, local replication of numerous pathogens and thereby delay their systemic spread. Second, neutrophils release an array of cytokines and chemokines and attract other cells of the innate as well as the acquired immune system [7–11]. Neutrophils therefore not only contribute to immediate pathogen restriction but also focus the specific immune response to the site of infection, which ultimately achieves control of the pathogen.

The role of neutrophils in pathogen control in vivo has been recently rendered accessible for investigation by the neutrophil depletion regimen by using the monoclonal antibody (MAb) RB6-8C5 [12]. Studies in neutrophil-depleted mice have revealed the importance of neutrophils for the initial control of a variety of pathogens. Neutrophils crucially contribute to the restriction of extracellular and intracellular bacteria [13–25], as well as to the control of certain viral and fungal infections [26, 27].

Mycobacterium tuberculosis is a major health problem, killing 2 million people every year [28]. Mononuclear phagocytes and T cells are crucially involved in the control and local containment of infection [29]. Less is known about the contribution of neutrophils to the control of *M. tuberculosis*. Neutrophils immigrate quickly to the site of mycobacterial entry [30 and present authors, unpublished observation], and they are found in granulomas after infection with *M. tuberculosis* [31]. However, conflicting in vitro evidence exists on the question of whether neutrophils are able to kill *M. tuberculosis* and hence actively contribute to growth restriction [32, 33]. We therefore investigated whether neutrophils participate in the initial control of *M. tuberculosis* in vivo by taking advantage of neutrophil-depleted mice. Comparisons with infections caused by other intracellular bacteria have confirmed that neutrophils are critical for the control of fast-replicating intracellular bacteria such as *Listeria monocytogenes* and *Salmonella typhimurium* [18, 20, 21, 34]. However, neutrophils apparently do not play an important role in the initial control of mycobacteria, independent of the replication rate and virulence of the species investigated. Infections with *M. tuberculosis, M. bovis* bacille Calmette-Guérin (BCG), and *M. fortuitum* were similarly controlled in neutrophil-depleted and control mice. This inefficiency of one of the most important lines of innate immune defense emphasizes the high priority of novel vaccination strategies that ensure a prompt antimycobacterial host response to partly compensate for the ineffective innate immune response.

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Materials and Methods

Mice. C57BL/6 mice were bred in the animal facilities of the Max-Planck-Institut at the Bundesamt für gesundheitlichen Verbraucherschutz und Veterinärmedizin in Berlin under specific pathogen-free conditions. Sex- and age-matched mice were used for all experiments under conventional housing conditions.

Bacteria. *L. monocytogenes* EGD Sv 1/2a was originally obtained from G. B. Mackaness and cultured in tryptic soy broth (Difco, Detroit) overnight at 37°C on a rocking platform and directly used for infection experiments. *S. typhimurium* SL1344 [35] was grown in Luria-Bertani (LB) broth (Difco). To ensure invasiveness, *S. typhimurium* was grown in high-osmolarity conditions. A single colony was inoculated into 5 mL of LB broth containing 0.3 *M* NaCl and was grown overnight as standing culture. This culture was diluted 1 : 100, grown to late-logarithmic phase (OD_{600} of 0.3–0.4), and directly used for infection experiments. *M. tuberculosis* strain Erdman and strain CDC 1551 [36], *M. bovis* BCG (Copenhagen), and *M. fortuitum* (generous gift of K. Feldmann, Gauting, Germany) were grown in Middlebrook 7H9 broth (Difco) supplemented with albumin-dextrose complex containing 0.05% Tween 80 and were stored in aliquots at −70°C. Mice were infected intravenously (iv) in the lateral tail vein with 10^3 cfu of *L. monocytogenes* or *S. typhimurium* or with 10^6 cfu of mycobacteria. Survival was monitored twice daily. For the determination of bacterial titers, organ homogenates were diluted in PBS for *L. monocytogenes* and *S. typhimurium* or PBS containing 0.05% Tween 80 for mycobacteria. Dilutions were plated on LB plates for *L. monocytogenes* and *S. typhimurium* and on Middlebrook 7H11 agar plates supplemented with oleic acid albumin-dextrose complex (Difco) for mycobacteria and were incubated at 37°C.

Antibody and depletion procedure. The neutrophil-depleting MAb RB6-8C5 was a kind gift from Dr. Robert Coffman [12]. The hybridoma RB6-8C5 was grown in culture and MAb affinity–purified over a Protein G-column (Pharmacia, Uppsala, Sweden) before use. For in vivo neutrophil depletion, mice were treated intraperitoneally (ip) with 100 μg of MAb RB6-8C5 1 day before infection. Depletion was verified by immunohistochemistry, blood smear, cytospin, and fluorescence-activated cell sorter (FACSCalibur; Becton Dickinson, San Jose, CA) analysis.

ELISA. Immuno-Maxi Sorp ELISA plates (Nunc, Wiesbaden, Germany) were coated overnight with 300 ng of MAb RB6-8C5 per well. Serial 3× dilutions of 10× prediluted mouse sera were detected by MAb alkaline phosphatase–conjugated goat anti-mouse immunoglobulin (TAGO, Burlingame, CA). The substrate for the color reaction was *p*-nitrophenyl-phosphate disodium (Sigma, St. Louis), and the color reaction was monitored at 405 nm (ELISA reader SpectraMAX 250; Molecular Devices, Sunnyvale, CA).

Immunohistochemistry. Freshly removed organs were immersed in Tissue-Tek O.C.T. (Miles, Elkhart, IN) and snap frozen in liquid nitrogen. Tissue sections of 5-μm thickness were cut in a cryostat, placed on siliconized glass slides, air-dried, fixed with acetone for 10 min, and stored at −70°C. Rehydrated tissue sections were incubated with primary rat MAb specific for: neutrophils (PMN: RB6-8C5 [12]), marginal zone metallophilic macrophages (MM: MOMA-1; Biomedicals, Augst, Switzerland), red pulp macrophages (RPM: F4/80; ATCC HB-198), $CD4^+$ T cells (CD4: YTS191 [37]) or $CD8^+$ T cells (CD8: YTS169 [37]). Primary rat MAbs were detected by a 2-fold sequential incubation with goat anti-rat immunoglobulin (Caltag, Burlingame, CA) and alkaline phosphatase–conjugated donkey anti-goat immunoglobulin (Jackson Lab, West Grove, PA). CD11c on dendritic cells was stained with the hamster MAb HL3 (Pharmingen, San Diego). Primary hamster immunoglobulin was detected by alkaline phosphatase–labeled rabbit anti-hamster immunoglobulin (Pharmingen) followed by alkaline phosphatase–labeled goat anti-rabbit immunoglobulin. Secondary and tertiary MAbs were diluted in Tris-buffered saline (TBS, pH 7.4) containing 5% normal mouse serum. All other dilutions were made in TBS alone. Incubations were done at room temperature for 30–40 min; TBS was used for all washing steps. Alkaline phosphatase was visualized by using naphthol AS-BI phosphate (Sigma) and New Fuchsin (Merck, Darmstadt, Germany) as substrate. Endogenous alkaline phosphatase activity was blocked by levamisole (Sigma). All color reactions were performed in the dark at room temperature for 15 min. Sections were counterstained with hemalum (Merck). Coverslips were mounted with glycerol/gelatin.

Phagocytosis and respiratory burst assays. Assays for phagocytosis and respiratory burst were performed according to the manufacturer's instructions (Orpegen Pharma, Heidelberg, Germany). In brief, for the phagocytosis assay, 100 μL of heparinized murine or human blood was mixed with 2×10^7 fluorescein isothiocyanate (FITC)–labeled *M. bovis* BCG or *L. monocytogenes* and incubated at 37° C for 10 min. Control samples were incubated on ice for 10 min. Nonphagocytosed bacteria were quenched with trypan blue. Cells were permeabilized, fixed, and stained with propidium iodide. For FACS analysis, eukaryotic cells were gated for high propidium iodide stain. Neutrophils and monocytes were gated according to forward and sideward scatter, and FITC fluorescence was monitored. For the burst assay, 100 μL of heparinized murine or human blood was mixed with 2×10^7 unlabeled *M. bovis* BCG or *L. monocytogenes* and incubated with di-hydro-rhodamine 123 at 37°C for 10 min. Control samples were mixed with medium and di-hydro-rhodamine 123 only. Cells were permeabilized, fixed, and stained with propidium iodide. For FACS analysis, eukaryotic cells were gated for high propidium iodide stain. Neutrophils and monocytes were gated according to forward and sideward scatter, and FITC fluorescence was monitored.

Results

Specific depletion of neutrophils by treatment with MAb RB6-8C5. To investigate the role of neutrophils in the control of mycobacterial infections, C57BL/6 mice were depleted of neutrophils by ip treatment with 100 μg of the MAb RB6-8C5 [12]. Immunohistochemical stainings of spleen sections from treated and untreated mice were compared 24 h later. Treatment with the MAb RB6-8C5 led to complete depletion of neutrophils from the spleen (figure 1*A,* 1*B*). Neutrophil depletion was specific because there was no difference in the immunohistochemical staining observed between MAb RB6-8C5–treated and untreated control mice for red pulp macrophages (figure 1*C,* 1*D*), marginal zone metallophilic macrophages (figure 1*E,* 1*F*),

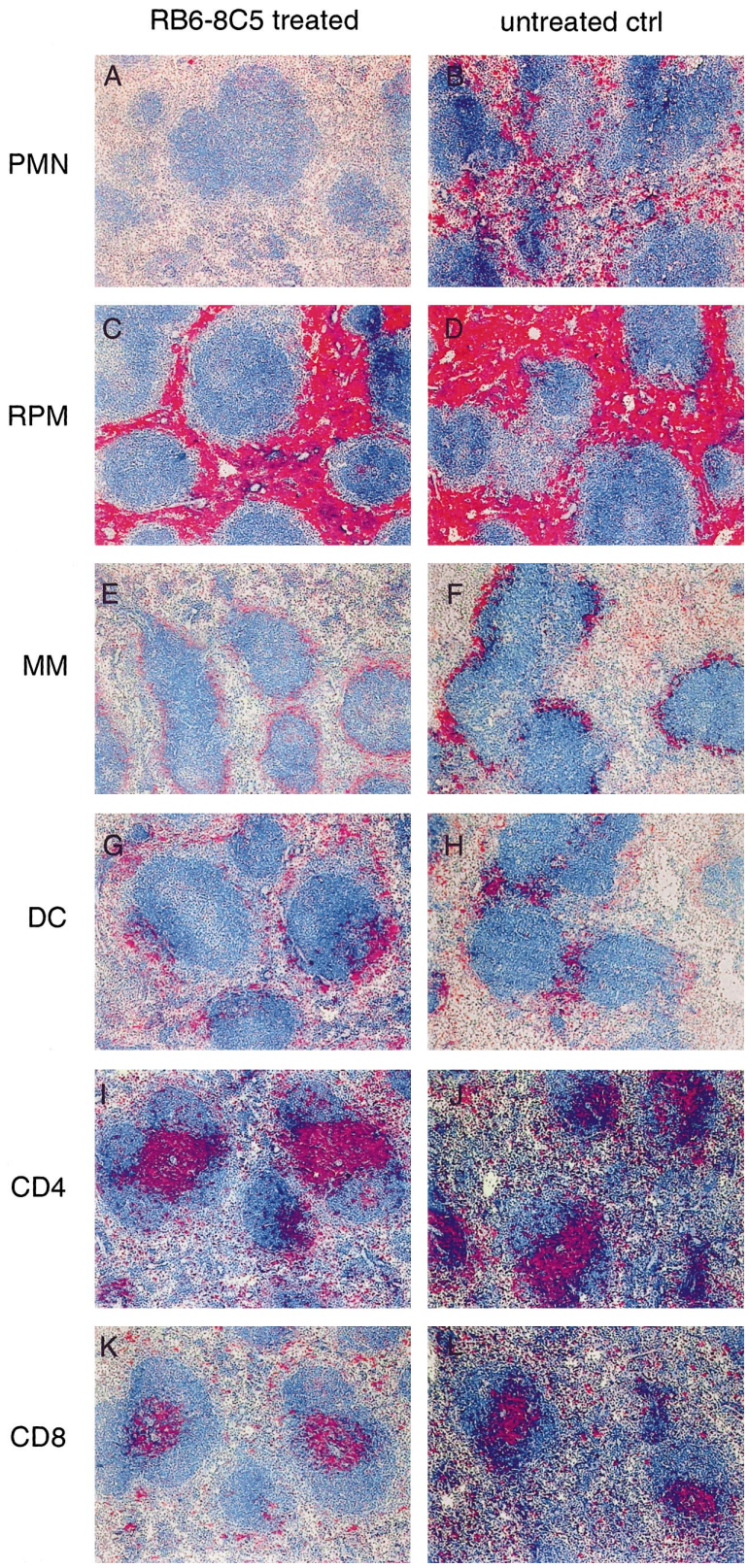

Figure 1. Depletion of splenic neutrophils after treatment with monoclonal antibody (MAb) RB6-8C5. C57BL/6 mice were treated intraperitoneally with 100 μg of MAb RB6-8C5 at day -1 *(A, C, E, G, I,* and *K)* or were left untreated *(B, D, F, H, J,* and *L)*. Spleens were removed at day 0, and frozen sections were immunohistochemically stained for neutrophils (PMN, MAb RB6-8C5) *(A* and *B),* red pulp macrophages (RPM, MAb F4/80) *(C* and *D),* marginal zone metallophilic macrophages (MM, MAb MOMA-1) *(E* and *F),* CD11c$^+$ dendritic cells (DC, MAb HL3) *(G* and *H),* CD4$^+$ T cells (CD4, MAb YTS191) *(I* and *J),* and CD8$^+$ T cells (CD8, MAb YTS169) *(K* and *L)*. Histological examinations were performed on 6 animals per condition. ctrl, control. Original magnification was $\times$100.

$CD11c^+$ dendritic cells (figure 1*G,* 1*H*), $CD4^+$ T cells (figure 1*I,* 1*J*), or $CD8^+$ T cells (figure 1*K,* 1*L*). In one experiment, control C57BL/6 mice were treated with an isotype-matched control MAb. No difference was observed in comparison with untreated control mice (data not shown). Therefore, in all subsequent experiments, control mice were left untreated. Completeness and specificity of neutrophil depletion were further confirmed by FACS analysis and morphological assessment using blood smear and cytospin preparations: neutrophils were efficiently depleted from spleen and blood. No other cell type was affected by the ip treatment with 100 μg of MAb RB6-8C5. Higher doses of MAb RB6-8C5 led to a reduction of $CD8^+$ T cells and did not improve depletion of neutrophils (2 mice were tested per condition; data not shown). Therefore, 100 μg of MAb RB6-8C5 was used in all subsequent experiments.

To deplete neutrophils for a prolonged period of time, mice were treated ip repetitively with 100 μg of MAb RB6-8C5 at 3-day intervals, and spleen sections were analyzed for the presence of neutrophils by immunohistochemistry. As shown in figure 2*A,* neutrophil depletion lasted for 3–4 days, independent of whether mice had been treated once or twice with MAb RB6-8C5. Spleens were repopulated with neutrophils by day 6 after treatment. Repetitive treatment did not lead to prolonged neutrophil depletion, most probably because of a strong antibody response against the rat MAb RB6-8C5. Sera of MAb RB6-8C5–treated mice exhibited a strong antibody response against the depleting antibody that was already detectable by ELISA between days 3 and 6 after treatment (figure 2*B*). Because repetitive treatment with MAb RB6-8C5 did not prolong neutrophil depletion, mice were treated once with 100 μg of MAb RB6-8C5 in all subsequent experiments.

Survival of neutrophil-depleted mice after infection with intracellular bacteria. To investigate the impact of neutrophils on control of infection with intracellular bacteria, mice were treated ip with 100 μg of MAb RB6-8C5 24 h before infection or mice were left untreated. Mice were infected iv with 10^3 cfu of *S. typhimurium* or *L. monocytogenes* or 10^6 cfu of *M. tuberculosis* strain Erdman, *M. tuberculosis* strain CDC 1551, *M. bovis* BCG, or *M. fortuitum,* and survival was monitored. A marked reduction in survival time of MAb RB6-8C5–treated mice compared with untreated control mice was observed after infection with the fast-replicating intracellular bacteria *S. typhimurium* and *L. monocytogenes* (figure 3*A,* 3*B*). In contrast, depletion of neutrophils during the initial phase of the immune response did not affect survival rate of mice after mycobacterial infection, independent of the virulence and replication rate of the bacterial species or strain tested (figure 3*C*–3*F*). Mice infected with *M. tuberculosis* strain Erdman or CDC 1551 started to die beginning from day 180 postinfection, irrespective of initial neutrophil depletion (data not shown).

Development of bacterial titers in neutrophil-depleted mice. The results presented in the previous paragraph are reflected in the development of bacterial load in various organs at different time points after infection. Three time windows were analyzed: early time points (10 min and 6 h) as a measure of the immediate filter function of neutrophils; intermediate time points (24 h and 48 h) as a measure of the bactericidal effector functions of neutrophils; and late time points (6 days and 21 days) as a measure of the impact of neutrophils on the overall immune balance. As shown in figure 4, mice treated with MAb RB6-8C5 had a higher bacterial burden than untreated control mice after infection with the fast-growing *S. typhimurium* and *L. monocytogenes.* In contrast, no substantial differences in bacterial numbers in the spleen, liver, and kidney were observed between MAb RB6-8C5–treated and untreated control mice after infection with slow-growing mycobacteria, although bacterial counts in the lung may have been somewhat higher in the treated animals (figure 5*A*–5*L*). However, after infection with fast-growing *M. fortuitum*, bacterial load in the lung was increased by 1–2 $\log_{10}$ in neutrophil-depleted mice compared with untreated control mice on day 7 (figure 5*O*).

Phagocytic activity and activation of neutrophils. Because neutrophil depletion during the initial phase of infection had no impact on the control of mycobacteria, the capacity of neutrophils to phagocytose mycobacteria was investigated. FITC-labeled *M. bovis* BCG or *L. monocytogenes* were incubated with peripheral blood from C57BL/6 mice at 37°C for 10 min. After quenching of nonphagocytosed bacteria, cells were analyzed by FACS for ingested bacteria after gating on living neutrophils and monocytes by forward/sideward scatter. After a 10-min incubation, neutrophils had already phagocytosed *M. bovis* BCG as well as *L. monocytogenes,* whereas monocytes had not yet phagocytosed the bacteria (figure 6*A*). Phagocytosis of *M. bovis* BCG was at least as efficient as phagocytosis of *L. monocytogenes,* although direct comparison was difficult, because the fluorescence intensity of FITC-labeled *M. bovis* BCG was much higher than that of FITC-labeled *L. monocytogenes,* as revealed by fluorescence microscopy (data not shown). Phagocytosis of *M. bovis* BCG and *L. monocytogenes* by human neutrophils was similar and also not detectable by human blood monocytes after 10 min of incubation with bacteria (figure 6*B*). After 30 min of incubation, blood monocytes had started to phagocytose *M. bovis* BCG and *L. monocytogenes,* whereas neutrophils had begun to undergo apoptosis, as revealed by loss of propidium iodide stain (data not shown).

Because neutrophils efficiently phagocytosed mycobacteria, activation of neutrophils upon phagocytosis of *M. bovis* BCG and *L. monocytogenes* was compared. Mouse and human peripheral blood leukocytes were incubated with *M. bovis* BCG or *L. monocytogenes* in the presence of fluorochrome di-hydro-rhodamine 123. Upon oxidation, di-hydro-rhodamine 123 turns into the fluorescent mono-hydro-rhodamine as a measurement for the production of reactive oxygen and nitrogen intermediates [38–44]. As shown in figure 7, mouse and, more evidently, human neutrophils exhibited considerably less fluorescent

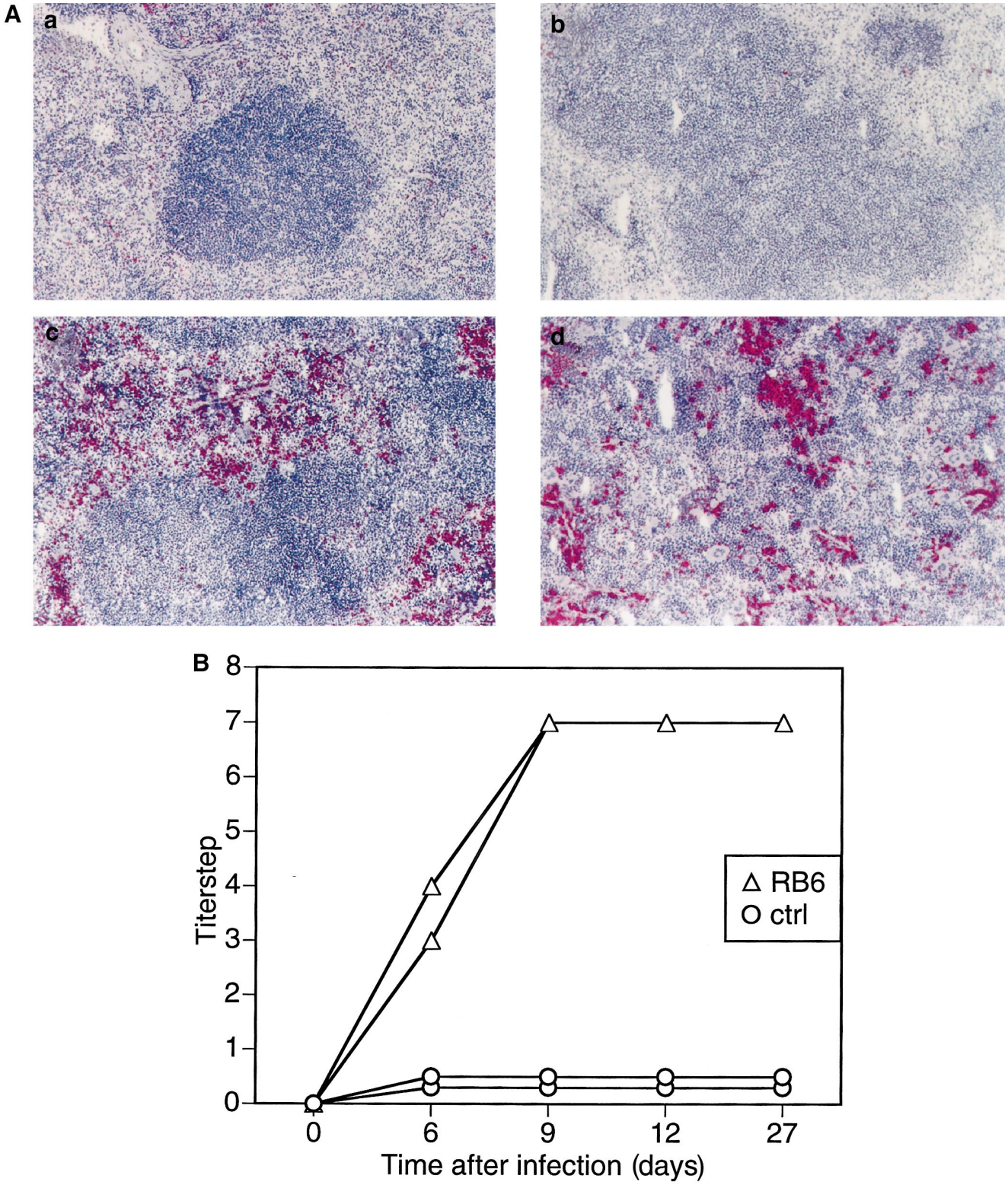

Figure 2. *A,* Depletion of splenic neutrophils after single or repetitive treatment with monoclonal antibody (MAb) RB6-8C5. C57BL/6 mice were treated intraperitoneally (ip) with 100 μg of MAb RB6-8C5 once at day 0 *(a* and *c)* or twice at days 0 and 3 *(b* and *d)*. Spleens were removed at day 3 *(a* and *b)* or day 6 *(c* and *d),* respectively, and frozen sections were immunohistochemically stained for neutrophils. Original magnification, $\times$100. *B,* Antibody response against MAb RB6-8C5. Serum samples from mice treated ip with 100 μg of MAb RB6-8C5 at days 0 and 3 (△) or from untreated control (ctrl) mice (○) were collected at the indicated time points and analyzed by ELISA for antibodies against MAb RB6-8C5. Titer steps represent 3$\times$ dilutions of 10$\times$ prediluted sera. Values represent individual mice. Shown is 1 representative out of 2 experiments.

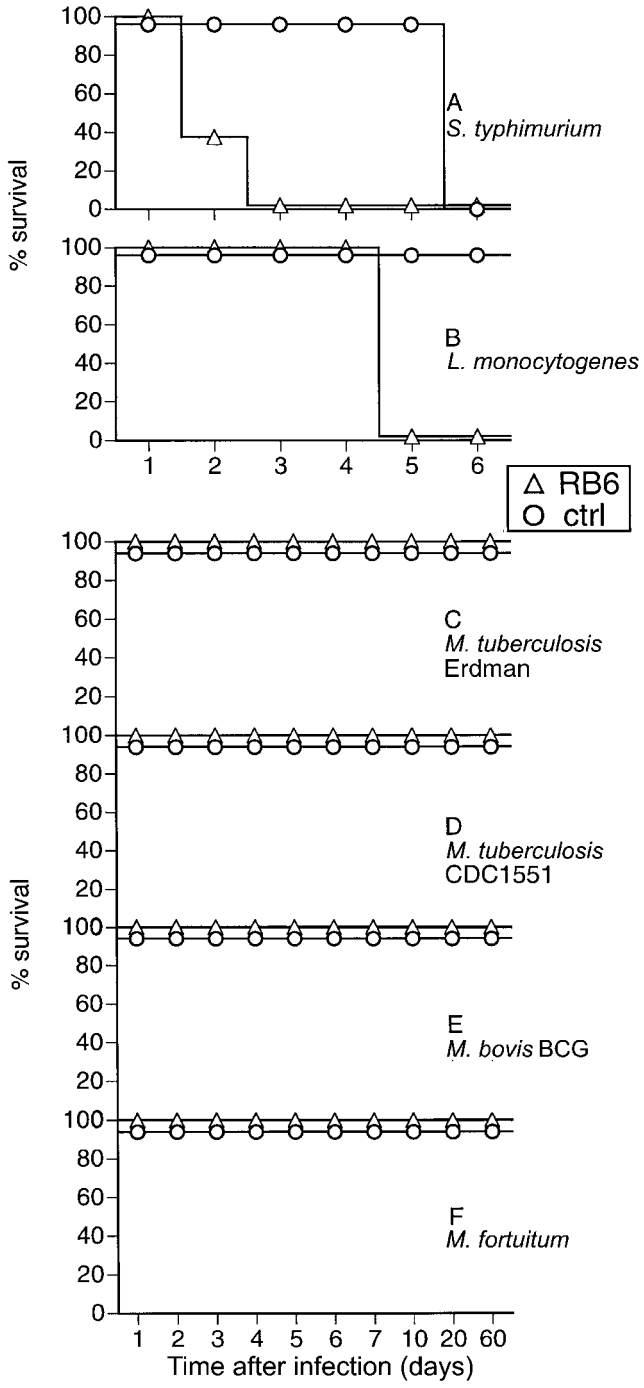

Figure 3. Survival of neutrophil-depleted mice after infection with intracellular bacteria. C57BL/6 mice were treated intraperitoneally with 100 μg of monoclonal antibody (MAb) RB6-8C5 at day −1 (△) or were left untreated (○). At day 0, mice were infected intravenously with 10^3 cfu of *Salmonella typhimurium (A)* or *Listeria monocytogenes (B)* or 10^6 cfu of *Mycobacterium tuberculosis* strain Erdman *(C)* or strain CDC 1551 *(D)*, *M. bovis* bacille Calmette-Guérin *(E)*, or *M. fortuitum (F)*, respectively, and survival was monitored. Each experiment consisted of 5 mice per group; shown is 1 representative out of 2 experiments.

mono-hydro-rhodamine after phagocytosis of *M. bovis* BCG than after phagocytosis of *L. monocytogenes,* indicating less production of reactive oxygen and nitrogen intermediates after infection by *M. bovis* BCG. This suggests that slow-growing *M. bovis* BCG activated neutrophils less efficiently than did *L. monocytogenes*.

Discussion

In the present study, neutrophil depletion of mice revealed different impacts on the control of fast-replicating versus slow-replicating intracellular bacteria. Whereas innate control of infection with fast-replicating *S. typhimurium* and *L. monocytogenes* depended on neutrophils, control of slow-replicating *M. tuberculosis* and *M. bovis* BCG was independent of neutrophils under the conditions used in this study. An intermediate phenotype was observed after infection with the fast-replicating, avirulent *M. fortuitum*. Survival rates were not influenced by neutrophil depletion; however, increased bacterial numbers in the lung were observed.

The in vivo depletion of neutrophils in mice was achieved by treatment with the MAb RB6-8C5. This MAb has been used

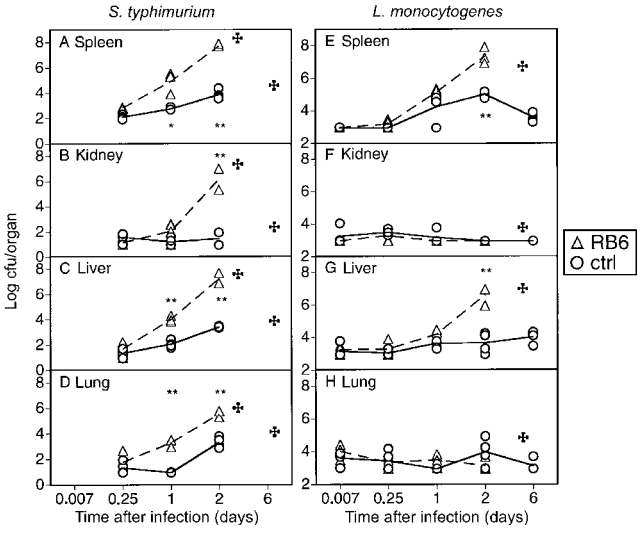

Figure 4. Bacterial load in neutrophil-depleted mice infected with fast-replicating *Salmonella typhimurium* and *Listeria monocytogenes*. C57BL/6 mice were treated intraperitoneally with 100 μg of monoclonal antibody (MAb) RB6-8C5 at day −1 (△) or were left untreated (○). At day 0, mice were infected intravenously with 10^3 cfu of *S. typhimurium (A–D)* or with 10^3 cfu of *L. monocytogenes (E–H)*. Bacterial numbers in organs were determined at the indicated time points. Values are log cfu per organ of individual mice; lines represent mean cfu per group of MAb RB6-8C5–treated mice *(dotted lines)* and of control (ctrl) mice *(solid lines)*. ⌘ Mice were dead by this time point. Each experiment consisted of 4 mice per group. The detection limit of the assay is log cfu = 1. Shown is 1 representative of 2 experiments. Values were compared using a 2-tailed Student's *t* test. Asterisks indicate statistically significant differences (*$P < .01$; **$P < .001$).

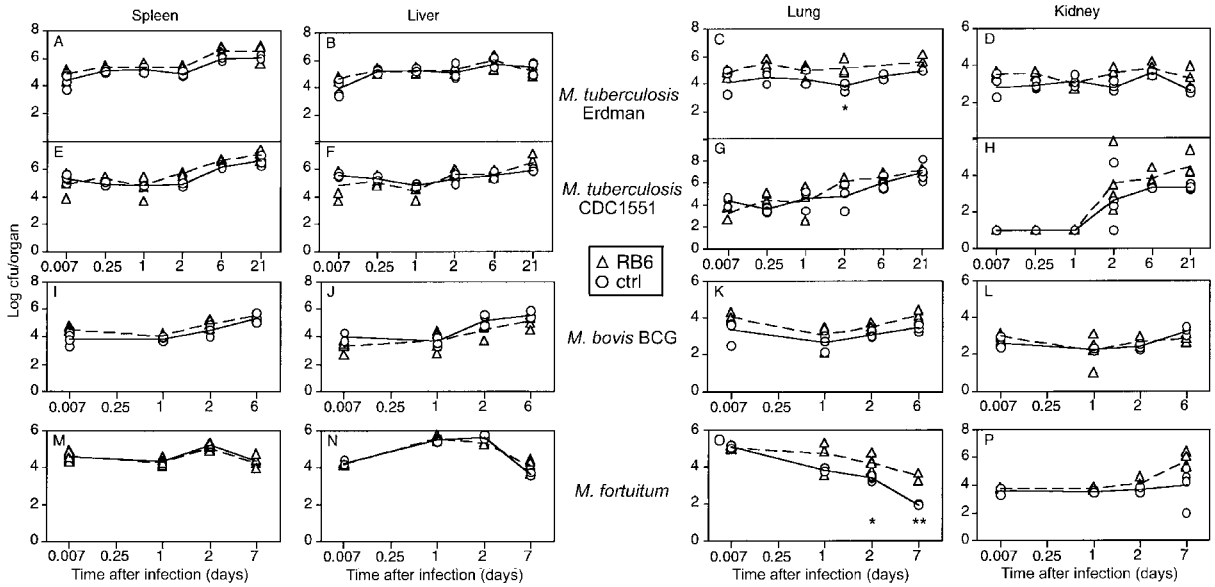

Figure 5. Bacterial load in neutrophil-depleted mice infected with mycobacteria. C57BL/6 mice were treated intraperitoneally with 100 μL of monoclonal antibody (MAb) RB6-8C5 at day −1 (△) or were left untreated (○). At day 0, mice were infected intravenously with 10^6 cfu of *Mycobacterium tuberculosis* strain Erdman *(A–D)* or strain CDC 1551 *(E–H), M. bovis* bacille Calmette-Guérin *(I–L),* or *M. fortuitum (M–P)*. Bacterial numbers in organs were determined at the indicated time points. Values are log cfu per organ of individual mice. Lines represent mean cfu per group of MAb RB6-8C5–treated mice *(dotted lines)* and of control (ctrl) mice *(solid lines)*. Each experiment consisted of 4 mice per group. The detection limit of the assay is log cfu = 1. Shown is 1 representative of 2 experiments. Values were compared by using a 2-tailed Student's *t* test. Asterisks indicate statistically significant differences (*$P < .01$; **$P < .001$).

for studying the contribution of neutrophils to the control of a variety of pathogens [14–27, 34]. Treatment protocols differed in dose and frequency of MAb application. A possible cross-depletion of other leukocyte subsets, especially of $CD8^+$ T cells, has been reported after high dose or repetitive treatment with MAb RB6-8C5 [22, 25, 26]. Since $CD8^+$ T cells play an important role in the control of *M. tuberculosis* [29, 45–47], we tested the MAb application protocol for specificity of neutrophil depletion. In our studies, the single ip treatment with 100 μg of MAb RB6-8C5 led to efficient and specific neutrophil depletion. However, higher doses or repetitive MAb treatment did not prolong the neutrophil depletion, compared with single treatment, but partially depleted $CD8^+$ T cells, as judged by immunohistochemistry and FACS analysis (data not shown). Our study therefore focused on the contribution of neutrophils to the antibacterial immune defense during the initial phase of the infection. Considering the problems caused by repetitive MAb RB6-8C5 treatment, protocols for long-term neutrophil depletion remain to be established very carefully.

The observed role of neutrophils in efficient control of infection with *S. typhimurium* and *L. monocytogenes* complements published literature [18, 20, 21]. In extension, the present study indicates that transient (3–4 days) neutrophil depletion of mice had no impact on infections with slow-growing mycobacteria. Although the existing in vitro evidence [32, 33] is conflicting on whether neutrophils contribute to the control of *M. tuberculosis* and by which mechanisms, our data indicate that in vivo, neutrophils do not contribute to the initial restriction of *M. tuberculosis*. Although neutrophils were highly efficient in the phagocytosis of mycobacteria, the respiratory burst, a measure of neutrophil activation, was smaller after phagocytosis of *M. bovis* BCG than after phagocytosis of *L. monocytogenes*. Possibly, mycobacteria partially suppressed activation of neutrophils and their subsequent oxidative and non-oxidative microbicidal activity. This is in agreement with the finding that several mycobacterial components interfere with activation of mononuclear phagocytes [48, 49]. Also, taking into account the short life span of neutrophils [10], a delayed activation of neutrophils may allow slow-growing mycobacteria to survive neutrophil effector functions.

Slow-growing mycobacteria exhibited a general resistance against murine neutrophil activity, independent of their virulence for humans. No differences in survival rate or development of bacterial numbers were observed in neutrophil-depleted mice after infection with the virulent *M. tuberculosis* strains Erdman or CDC 1551 when compared with the nonvirulent *M. bovis* BCG. Furthermore, no difference was observed after infection with the *M. tuberculosis* laboratory strain Erdman and the clinical isolate CDC 1551 [36]. This clinical isolate has been identified as the cause of an *M. tuberculosis* outbreak because of its high transmission rate. To the extent that murine and human neutrophils are functionally comparable, our data sug-

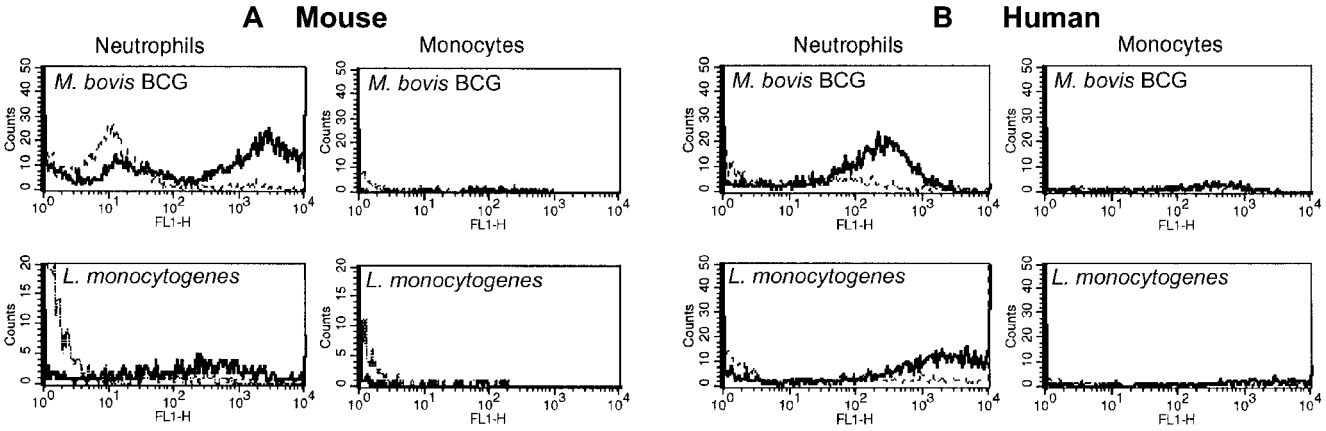

Figure 6. Phagocytosis of intracellular bacteria by neutrophils. *(A)* 100 μL of mouse or *(B)* 100 μL of human heparinized peripheral blood was incubated with 2×10^7 fluorescein isothiocyanate (FITC)–labeled *Mycobacterium bovis* bacille Calmette-Guérin or FITC-labeled *Listeria monocytogenes* at 37°C for 10 min. Phagocytosis of bacteria was detected by FACS analysis after gating on viable neutrophils or monocytes by forward/sideward scatter. Dotted lines represent control incubation at 0°C. Shown is 1 representative experiment of 3.

gest that the elevated transmission rate was not because of increased resistance against neutrophil microbicidal activities.

In the present study, no impact of neutrophils during the initial phase of immune defense against slow-growing mycobacteria was observed, either at early or at late stages of infection. Results from early and intermediate time points exclude filter function and direct microbicidal effector function of neutrophils, respectively. Results from late time points of infection indicate that neutrophil depletion did not influence the balance between immune response and bacterial kinetics. This might be because of the following reasons: (1) the balance can be maintained redundantly by other members of the innate immune system, such as tissue macrophages, or (2) the balance is not critically shifted due to the short duration of the neutrophil depletion.

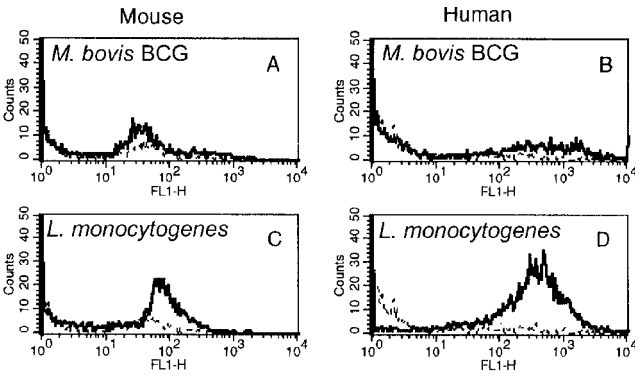

Figure 7. Neutrophil activation for respiratory burst upon phagocytosis of intracellular bacteria. One hundred microliters of mouse *(A* and *C)* or human *(B* and *D)* heparinized peripheral blood was mixed with 2×10^7 cfu of *Mycobacterium bovis* bacille Calmette-Guérin *(A* and *B)* or *Listeria monocytogenes (C* and *D)* and incubated with di-hydro-rhodamin 123 (Orpegen, Heidelberg, Germany) at 37°C for 10 min. Oxidation of di-hydro-rhodamin 123 by neutrophil-induced ROI and RNI after phagocytosis of bacteria was detected by fluorescence-activated cell sorter analysis after gating on viable neutrophils by forward/sideward scatter. Dotted lines represent control incubation at 37°C with medium and di-hydro-rhodamin 123 only. Shown is 1 representative experiment of 3.

The findings of the present study are consistent with observations from neutropenic patients. Neutropenia can result from exposure to x-rays or chemotherapy or can result from inherited genetic defects [11, 50–54]. Neutropenic patients reflect a more chronic neutropenia than do mice experimentally depleted of neutrophils. Nevertheless, patients mainly are infected with fast-replicating bacteria and fungi [11, 51–53, 55]. Few reports described infection of neutropenic patients with *M. fortuitum*, which may reflect our observation of increased *M. fortuitum* burden in the lung of neutrophil-depleted mice. No reports are available to demonstrate an increased susceptibility of neutropenic patients toward *M. tuberculosis*. The seemingly neutrophil-independent control of *M. tuberculosis* infection in non-neutropenic patients, therefore, might be caused by a partial suppression of neutrophil activation mediated by mycobacteria, as suggested by the results in the present study. However, further investigations are needed to clarify how such suppression is achieved.

In summary, the dual function of neutrophils, immediate pathogen restriction and local focusing of the specific immune response, is vital for the control of fast-replicating intracellular bacteria but apparently subordinate for the control of slow-replicating intracellular bacteria. This emphasizes the importance of the development and improvement of an effective vaccination strategy against *M. tuberculosis,* enabling the memory type of the acquired immune response to overcome the insufficient innate immune response mediated by neutrophils.

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